TWIN AND EPIDEMIOLOGICAL STUDIES ON INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1, RELATIONSHIPS TO INSULIN SENSITIVITY AND CARDIOVASCULAR RISK

Maija Harrela

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Department of Obstetrics and Gynaecology,

Helsinki University Central Hospital,

University of Helsinki, Finland

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ACADEMIC DISSERTATION

To be presented with the permission of the Medical Faculty of the University of Helsinki for public criticism in the Auditorium of the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Haartmaninkatu 2, Helsinki, on 17 May 2002 at 12 noon.

Supervised by

Professor Markku Seppälä
Department of Obstetrics and Gynaecology,
Helsinki University Central Hospital
University of Helsinki, Finland

Professor Jaakko Tuomilehto
Diabetes and Genetic Epidemiology Unit,
National Public Health Institute, Helsinki, Finland

Pekka Leinonen, MD
Department of Obstetrics and Gynaecology,
Helsinki University Central Hospital,
University of Helsinki, Finland

Reviewed by

Docent Timo Strandberg
Department of Medicine,
Helsinki University Central Hospital,
University of Helsinki, Finland

Docent Hannu Martikainen
Departmant of Obstetrics and Gynaecology,
Oulu University Central Hospital
University of Oulu, Finland

Opponent

Professor Juha Tapanainen
Department of Obstetrics and Gynaecology,
Oulu University Central Hospital,
University of Oulu, Finland

To Jari, Irene and Kirmo

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals I-IV.

I Harrela M, Koistinen H, Kaprio J, Lehtovirta M, Tuomilehto J, Eriksson J, Toivanen L, Koskenvuo M, Leinonen P, Koistinen R, Seppälä M: Genetic and environmental components of interindividual variation in circulating levels of IGF-I, IGF-II, IGFBP-1, and IGFBP-3. J Clin Invest 98: 2612-2615, 1996.

II Harrela M, Koistinen R, Tuomilehto J, Nissinen A, Seppälä M: Low serum insulin-like growth factor-binding protein-1 is associated with an unfavourable cardiovascular risk profile in elderly men. Ann Med 32: 424-428, 2000.

III Harrela M, Qiao Q, Koistinen R, Tuomilehto J, Nissinen A, Seppälä M, Leinonen P: High serum insulin-like growth factor binding protein-1 is associated with increased mortality in elderly men. Horm Metab Res 34: 144-149, 2002.

IV Harrela M, Leinonen P, Groop L, Kaprio J, Tuomilehto J, Koistinen R, Seppälä M, Lehtovirta M: Insulin-like growth factor binding protein-1, insulin secretion and insulin sensitivity in twins. Submitted.

ABBREVIATIONS

BMI	body mass index
CVD	cardiovascular disease
CHD	coronary heart disease
D	Dalton
DZ	dizygotic
FPI	first-phase insulin
GH	growth hormone
HDL	high-density lipoprotein
HOMA	homeostasis model assessment
IFMA	immunofluorometric assay
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP-1	insulin-like growth factor binding protein-1
IGFBP-3	insulin-like growth factor binding protein-3
Il	interleukin
IVGTT	intravenous glucose tolerance test
kD	kiloDalton
LDL	low-density lipoprotein
LPI	late-phase insulin
MSA	multiplication-stimulating activity
NCEP	National Cholesterol Education Program
NIDDM	noninsulin-dependent diabetes mellitus
NSILA	nonsuppressible insulin-like activity
MZ	monozygotic
OR	odds ratio
PAPP-A	pregnancy-associated protein A
PCO	polycystic ovaries
TNF	tumor necrosis factor
VSMC	vascular smooth muscle cell
WHO	World Health Organization

1. ABSTRACT

Insulin-like growth factor binding protein-1 (IGFBP-1) belongs to a family of soluble IGFBPs which bind insulin-like growth factors and modulate their biological actions. Insulin down-regulates the IGFBP-1 production in the liver. The aim of the study was to examine the genetic influence on the regulation of the IGF system and the relation of circulating IGFBP-1 to cardiovascular risk factors, cardiovascular mortality, insulin secretion, and insulin sensitivity.

The magnitude of the genetic component in the variation of IGF-I, IGF-II, IGFBP-1, and IGFBP-3 in serum was assessed in twin pairs. No significant heritability was found for the IGFBP-1 concentrations. A negative correlation was found between serum IGFBP-1 and late-phase insulin secretion, and a positive correlation between IGFBP-1 and insulin-stimulated glucose uptake. Cross-twin cross-trait correlations between IGFBP-1 and insulin secretion were higher in monozygotic than dizygotic twins. No difference was seen between monozygotic twins and dizygotic twins in the correlation between IGFBP-1 and insulin-stimulated glucose uptake. In conclusion, serum IGFBP-1 is associated with insulin secretion and insulin sensitivity and appears to be regulated mainly by environmental factors. In addition a latent, possibly genetic, influencing factor may be shared by IGFBP-1 and late-phase insulin secretion but not by IGFBP-1 and insulin sensitivity.

Insulin resistance is thought to be the underlying abnormality leading to clustering of cardiovascular risk factors, i.e., glucose intolerance, hypertension, decreased serum high-density lipoprotein cholesterol, and elevated triglyceride concentration. Insulin resistance is counterbalanced by increased insulin secretion, which reduces hepatic IGFBP-1 production. In our cross-sectional analysis, we showed that low serum IGFBP-1 is associated with a cluster of cardiovascular risk factors. Low serum IGFBP-1 is obviously another sign of the metabolic syndrome. In the follow-up study, we unexpectedly found out that it was not the men with low IGFBP-1 who carried the highest risk for cardiovascular death. Men with high serum IGFBP-1 had an increased risk for coronary heart disease, cardiovascular and total mortality.

2. INTRODUCTION

Insulin-like growth factors (IGF I and IGF II) are polypeptides that participate in the growth and function of almost every organ in the body. The IGFs bear marked structural homology with insulin and proinsulin and exert growth-promoting, differentiative, and insulin-like metabolic effects (Froesch et al. 1985). Serum and tissue concentrations of IGF-I are regulated by growth hormone, nutritional status, age, and other factors (Baxter 1986). The stability, availability, and bioactivity of circulating IGFs are regulated by their receptors (Rechler and Nissley 1990) and binding proteins (Shimasaki and Ling 1991).

Serum insulin-like growth factor binding protein-1 (IGFBP-1) binds both IGFs and modulates their actions. Insulin down-regulates the production of IGFBP-1 in the liver (Suikkari et al. 1988), which is the major organ contributing to the circulating IGFBP-1 pool. The aim of the study was to examine the genetic influence on the regulation of serum IGF system and the the relation of circulating IGFBP-1 to cardiovascular risk factors, cardiovascular mortality, insulin sensitivity, and insulin secretion.

3. REVIEW OF THE LITERATURE

3.1. INSULIN-LIKE GROWTH FACTORS

Three different observations led to the discovery of insulin-like growth factors I and II. In 1957, Salmon and Daughaday demonstrated that growth hormone (GH) had no direct metabolic effect on skeletal tissue but induced factors in serum that mediated its metabolic effects. These factors were first called sulfation factors, according to their ability to stimulate the incorporation of radio-labeled sulfate into costal cartilage of hypophysectomized rats. The term somatomedins, mediators of somatotropin action, was introduced by Daughaday et al. in 1972. Other investigators found factors in serum that could exert insulin-like effects on tissues but could not be suppressed by the addition of anti-insulin antiserum, called nonsuppressible insulin-like activity NSILA-I and -II (Froesch et al. 1967). These substances were later renamed IGF-I and IGF-II (Rinderknecht and Humbel 1976). Purification and amino acid sequence determination of somatomedin-C (Klapper et al. 1983) and its deaminated form somatomedin-A (Enberg et al. 1984), have shown that they are identical to IGF-I. Several factors in serum have proliferative effect in cell cultures. One of them purified in a rat liver cell culture medium, has multiplication-stimulating activity (MSA), (Dulak and Temin 1973; Moses et al. 1980). MSA has 93% homology with human IGF-II (Rinderknecht and Humbel 1978b; Marquardt et al. 1981).

Structure and synthesis

IGF-I consists of 70 amino acids and has a molecular weight of 7649 D. It has 62% homology with IGF-II, which has 67 amino acids and a molecular weight of 7471 D. Substantial structural homology exists between the two IGFs and proinsulin: 20 of insulin´s 52 amino acids are identical to IGF-I and IGF-II. (Rinderknecht and Humbel 1976; 1978a,b)

IGF-I and IGF-II are products of single genes localized on the long arm of chromosome 12 and on the short arm of chromosome 11, respectively (Brissenden et al.1984; Tricoli et al. 1984). The IGF-II gene is contiguous with the insulin gene (Bell et al. 1985). Although the liver accounts for most of the circulating IGFs in adults, virtually all human tissues have been shown to synthesize IGF-I and IGF-II at some point during pre- or postnatal development (Rechler and Nissley 1990).

IGF Receptors

The IGFs exert their effects by binding to cell surface receptors. Two subtypes of IGF receptors have been identified. Type I IGF receptor is a glycoprotein which consists of two extracellular ligand-binding alpha subunits (125 kD) and two transmembranal beta subunits (95 kD) with intrinsic tyrosine kinase activity. Type I receptor has a high affinity for IGF-I and IGF-II but a much lower affinity for insulin (Rechler and Nissley 1985; Ullrich et al. 1986). Type II IGF receptor is a large monomeric transmembrane 250-kDa protein with a short sytoplasmic domain which lacks kinase activity (Morgan et al. 1987). IGF-II binds with high affinity to type II receptor, whereas IGF-I binds less avidly. Insulin has no affinity to type II receptor. Both type I and II receptors have been identified in a wide variety of tissues and cell cultures (Baxter 1986).

Biological effects and regulation

The IGFs act in an endocrine, paracrine, and autocrine manner in many tissues (Underwood et al. 1986a).The metabolic effects of IGFs are similar to but milder than the actions of

insulin in insulin target tissues (Froesch et al, 1985). These effects are mediated through insulin receptors in adipose tissue and through type I IGF receptor and insulin receptors in skeletal muscle cells. Long-term effects of IGFs on cell differentation and proliferation are mediated mainly through type II IGF receptors (Froesch et al. 1985). Effects of IGFs on cell replication and DNA synthesis have been demonstrated in many cell types (Froesch et al. 1985; Baxter 1986). IGF-I mediates the mitogenic actions of GH to cartilage tissue (Daughaday et al. 1972).

Serum IGF-I level is dependent on age. Levels are low at birth, rise progressively during childhood, and peak during midadolescence (Hall and Sara 1984). After adolescence, IGF-I concentrations fall (Clemmons and Van Wyk 1984). Serum IGF-II concentration reaches adult levels in early childhood and changes little with advancing age (Zapf et al. 1981). Serum IGF-I and IGF-II levels show no marked diurnal variation (Horner et al. 1981).

The association between GH and serum IGF-I level was one of the first clinical observations about these growth factors. GH deficiency is associated with a low serum IGF-I concentration. After intramuscular GH injection, there is an increase in serum IGF-I in GH-deficient patients as well as normal adults (Clemmons and Van Wyk 1984). Acromegaly is associated with increased serum IGF-I concentrations (Clemmons and Underwood 1986).

Nutritional intake is an important regulator of IGF-I but not of the IGF-II. The IGF-I levels are reduced in malnutrition (Underwood et al. 1986b) and in some chronic disorders such as chronic liver disease and primary hypothyroidism (Baxter 1986). IGF-I concentration rises during pregnancy (Furlanetto et al. 1978).

3.2. INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1

In 1988, insulin-like growth factor binding protein-1 (IGFBP-1) became the first characterized member of a group of structurally related soluble proteins, that specifically bind and modulate the actions of IGF-I and IGF-II (Brinkman et al. 1988; Lee et al. 1988; Julkunen et al. 1988).

IGFBP-1 protein structure and gene organisation

The IGFBP-1 amino acid sequence contains 12 N-terminal and 6 C-terminal cysteine residues that are conserved in other mammalian IGFBP-1 sequences and amongst other IGFBPs; both of the cysteine-rich regions are required for optimal IGF binding (Brinkman et al.1988; Julkunen et al. 1988). The nonconserved IGFBP-1 midregion may act as both a hinge which defines ligand-binding characteristics and as a specific target for protease activity. (Lee et al. 1993)

The IGFBP-1 protein can be divided into three regions according to cysteine clusters. Region 1 contains the first 79 residues of human IGFBP-1, including the 12 N-terminal cysteines, region 2 includes residues 80-144, and region 3 spans residues 145-234 containing the 6 C-terminal cysteines. Region 3 contains an Arg-Gly-Asp (RGD) sequence which is conserved in rat, cow, and mouse IGFBP-1 (Lee et al. 1997). The RGD sequences are present in a group of extracellular matrix proteins and mediate binding of these proteins to specific cell surface receptors known as integrins (Ruoslahti and Pierschbacher, 1987).

IGFBP-1 is secreted as a phosphoprotein (Koistinen et al. 1993) with serine residues at

positions 101, 169, and 119, serving as phosphorylation sites (Jones et al. 1993).

The IGFBP-1 gene has been localized to chromosome 7p14-p12 (Alitalo et al. 1989) and is contiguous with the IGFBP-3 gene (Ehrenborg et al. 1992). The IGFBP-1 gene is 5 kb long and contains four exons separated with three introns (Cubbage et al. 1989). In human adults, the IGFBP-1 gene is expressed primarily in the liver (Brinkman et al. 1988), secretory and decidualized endometrium (Rutanen et al. 1986) and ovarian granulosa cells (Jalkanen et al. 1989) The IGFBP-1 promoter region contains a binding site for hepatic nuclear factor 1 (HNF1), a DNA-binding protein which is primarily responsible for basal IGFBP-1 promoter activity in hepatoma cells (Suwanihkul et al. 1990). The promoter region also includes glucocorticoid responsive elements (GRE1 and GRE2) which confer the stimulatory effects of glucocorticoids to the IGFBP-1 promoter (Suwanihkul et al. 1994). The promoter region contains a binding site for hepatic nuclear factor 3 (HNF3). This element is also called insulin responsive element (IRE) as it confers the inhibitory effect of insulin on IGFBP-1 promoter activity (Suwanihkul et al. 1994). Proximally in the promoter region lies a cAMP-responsive element (CRE), which confers cAMP stimulation to the IGFBP promoter (Suwanihkul et al. 1993).

Regulation of IGFBP-1

Early investigations on human IGFBP-1 plasma levels described rapid daily fluctuations ranging 10-fold in normal, healthy individuals (Baxter and Cowell 1987). A similar phenomenon had been described in earlier studies of PP12 (Seppälä et al. 1983). Suikkari and coworkers (1988) and Brismar and associates (1988) showed that IGFBP-1 levels inversely correlated with insulin levels in fasting normal individuals, as well as in individuals with disorders of insulin secretion.

IGF-I and IGF-II inhibit IGFBP-1 expression in HepG2 human hepatoma cells, which possess abundant type I IGF receptors (Grissom et al. 1993), but not in normal human liver cells in which type I receptors are low or absent (Villafuerte et al. 1992).

Glucocorticoids and cAMP stimulate IGFBP-1 transcription (Unterman et al. 1993), but these effects are observed only in conditions of low insulin effect (Powell et al. 1993). Other stimulants include thyroid hormones (Angervo et al., 1993) and epidermal growth factor (Angervo et al. 1992). Cytokines may alter IGFBP-1 expression. IL-1beta, TNF alpha, and IL-6 stimulate IGFBP-1 production by HepG2 cells (Samstein et al. 1996).

Regulatory actions of IGFBP-1

IGFBP-1 inhibits IGF-mediated growth and differentiative functions in many tissues. Numerous *in vitro* studies have shown that IGFBP-1 inhibits IGF-I-induced cell proliferation in the ovary (Angervo et al. 1991), osteosarcoma cells (Campbell and Novak 1991), prostate (Figueroa et al. 1995), and fibroblasts (Okajima et al. 1993). *In vivo* studies are consistent with the inhibitory role on IGF-I action. Recombinant IGFBP-1 has been shown to inhibit somatic growth stimulated by IGF-I and growth hormone in hypophysectomized rats (Cox et al. 1994). Growth retardation and hyperglycemia has been demonstrated in transgenic mice that overexpressed IGFBP-1 (Rajkumar et al., 1995). This is not surprising since IGFBP-1 can bind IGFs with high affinity. However, under some circumstances, IGFBP-1 may enhance the mitogenic actions of IGF-I (Elgin et al. 1987; Koistinen et al. 1990).

In summary, IGFBP-1 acts as an endocrine factor regulating the bioavailability of IGF-

I and modulating IGF-mediated tissue metabolism. IGFBP-1 also acts as an autocrine/paracrine factor and appears to play a crucial role in the female reproductive system (Lee et al. 1997).

IGFBP-1 measurements

The first quantitative assays for IGFBP-1 were based on the principle of competitive displacement radioimmunoassay (RIA) (Rutanen et al. 1982). The development of monoclonal antibodies to IGFBP-1 led to the development of the dual-epitope, direct detection assay in which the antigen in the standards and unknowns is quantitatively bound to purified antibody, usually coated to a solid surface, and detected using a second antibody preparation which binds to a different site, or epitope, on the antigen (Koistinen et al. 1987). The dual-epitope method can easily be adapted to automated methods which employ fluorescent, electrochemical, or other detection methods (Koistinen et al. 1996).

Serum IGFBP-1 level shows variability inversely related to insulin secretion, and, therefore, fasting must preceed any routine IGFBP-1 sampling. A significant correlation exists between fasting serum IGFBP-1 and the mean of 24 hour IGFBP-1. (Brismar et al. 1995)

3.3. INSULIN-LIKE GROWTH FACTORS AND THEIR BINDING PROTEINS IN HUMAN GLUCOSE AND LIPID METABOLISM

Since IGFs exert 5-10% of the insulin-like activity of insulin and circulate at a concentration of about 1000 greater than insulin, the hypoglycemic potential of serum IGFs is vast. Most hypoglycemic activity is not expressed, being inhibited by the complexing of IGFs with IGFBP-3 and acid-labile subunit.(Baxter 1995)

Intravenous infusion of recombinant IGF in healthy volunteers suppresses insulin secretion and increases glucose disposal (Zenobi et al. 1992a). Several studies have demonstrated that the administration of rhIGF-I can reduce insulin resistance in healthy subjects as well as in subjects with severe insulin resistance (Schoenle et al. 1991; Hussain et al. 1993). Insulin infusion studies show a stimulation of IGF-I production and blocking of IGFBP-1 production, leading to increased free IGF-I (Brismar et al. 1994). Thus, IGF-I suppresses insulin secretion and insulin, in turn, stimulates IGF-I production in the liver.

rhIGF-I has been shown to decrease LDL cholesterol and triglyceride levels in subjects with type II diabetes (Zenobi et al. 1992b; Schalch et al. 1993).

The effects of IGF-II on glucose metabolism are not well defined, but animal studies suggest that it has insulin-like metabolic effects, although with a significantly lower potency (Burvin et al. 1998). In patients with IGF-II -secreting tumors, hypoglycemia may result from a failure of IGFBP-3 to adequately sequester the IGFs. In these patients, blood glucose levels correlated positively with IGFBP-3 levels and inversely with IGFBP-2 (Baxter 1995). Unlike the other IGF-binding proteins, IGFBP-1 is acutely regulated in circulation, in a manner consistent with its action as a glucose counterregulator (Baxter 1995).

Early clinical studies on IGFBP-1 indicated insulin dependency (Brismar et al. 1988; Suikkari et al. 1988). Subsequent investigations confirmed that insulin, via inhibition of IGFBP-1 transcription, is the primary determinant of IGFBP-1 expression (Lewitt and Baxter 1989). Insulin infusion studies with concurrent hepatic vein and peripheral vein/

artery sampling studies in patients with IDDM have shown a complete inhibition of hepatic IGFBP-1 production in 120 minutes, with a decline seen in 60 minutes (Brismar et al. 1994). Hyperinsulinemic obese subjects had low fasting serum IGFBP-1 concentrations which were inversely correlated with insulin. The inverse relationship between insulin and IGFBP-1 was similar in normal, normal-obese, and both obese and non-obese women with polycystic ovary (PCO) syndrome, indicating that the decreased IGFBP-1 levels seen in hyperinsulinemic obesity is due to hyperinsulinemia and not obesity per se. (Conover et al. 1992). It is known that a large number of obese PCO patients and a significant number of lean PCO patients are hyperinsulinemic (Morin-Papunen et al, 2000) and insulin resistant (Dunaif et al., 1989). However, since the cohort in our cardiovascular studies consists of elderly men, the literature on metabolic changes in PCOS is not covered in this review.

Hyperinsulininemia and insulin resistance are thought to be major factors in the pathogenesis of cardiovascular disease, and detection and treatment of these conditions could have individual and public health benefits. However, these metabolic abnormalities are often asymptomatic.

In 1993, the sex hormone binding globulin (SHBG) was suggested as marker for hyperinsulinemia and/or insulin resistance (Nesttler 1993). SHBG is a liver-derived protein regulated by sex steroids, T4, and insulin. Secretion of insulin to the portal vein exposes the liver to a high concentration of insulin, and low serum SHBG was reported to correlate with hyperinsulinemia (Preziosi et al. 1993) and insulin resistance (Birkeland et al. 1993). This provided a potential explanation for the observation that low serum SHBG was a predictor for type II diabetes mellitus (Lindstedt et al. 1991). However, in a prospective study with 760 men and 624 women, SHBG correlated with heart disease risk factors, but showed no association with cardiovascular disease or ischemic heart disease mortality over the 19-year follow-up (Goodman-Gruen 1996).

At the beginning of this study, we hypothetized that IGFBP-1, produced by the liver and down-regulated by insulin, could be a marker for hyperinsulinemia and/or insulin sensitivity. Later, reduced serum IGFBP-1 was shown to predict hyperinsulinism in obese women without NIDDM (noninsulin-dependent diabetes mellitus) (Mogul et al. 1996). In 1995, an important report was published, in which Yki-Järvinen and her group used an insulin-clamp protocol with healthy and IDDM (insulin-dependent diabetes mellitus) subjects to demonstrate that hepatic portal insulin concentrations regulate the production of both SHBG and IGFBP-1 in the liver. IGFBP-1 and SHBG can therefore be used as markers of insulin sensitivity only in individuals with intact insulin secretion. (Yki-Järvinen et al. 1995)

3.4. INSULIN-LIKE GROWTH FACTORS AND THEIR BINDING PROTEINS IN CIRCULATION

Serum IGF concentrations are 10 to 1000-fold higher than those of insulin, but most circulating IGFs are bound to insulin-like growth factor binding proteins and only about 1-10 % are free (Daughaday and Rotwein, 1989). Such free IGFs , though cleared rapidly, have the potential to exert as much insulin-like activity as insulin.

IGFBP-1 may have a role in inhibiting free IGF activity. IGFBP-1 differs from other IGFBPs, showing marked diurnal variation due to changing metabolic status. Levels rise more than 10-fold at fasting and fall after the morning meal (Yeoh 1988). Due to the great variability in IGFBP-1 levels resulting from its acute metabolic regulation, comparable

normal ranges in different laboratories have been difficult to establish (Baxter 1994). Serum IGFBP-1 concentration declines in adolescence (Argente et al. 1993) and remains low in adulthood. With advancing age, there may be a slight increase in serum IGFBP-1 as the inverse correlation with insulin level becomes less pronounced (Rutanen et al. 1993).

Hemodynamic actions of IGF-I

IGF-I has an effect on the regulation of vascular tone, causing an increase in skeletal muscle blood flow and a decrease in vascular resistance. In animal experiments, IGF-I may have differential effects in different vascular beds, exerting a vasodilatory effects on rat aortic ring and vasoconstrictor effects in rat mesenteric artery (Wu et al. 1994). In men recombinant human IGF-I increases forearm blood flow (Copeland and Nair 1996).

Nitric oxide (NO) in the vascular effects of IGF-I

In 1991, Haylor and his colleagues demonstrated that nitric oxide synthesis inhibitor prevented vasodilatation by IGF-I in the rat renal artery. Later, IGF-I was shown to stimulate NO production in human vascular endothelial cells (Tsukahara et al. 1994) and vascular smooth muscle cells (Muniyappa et al. 1997). In healthy volunteers, an IGF-I-mediated increase in forearm blood flow could be blocked by an inhibitor of nitric oxide synthase (Fryburg 1996). IGF-I has been shown to induce coronary vasorelaxation (Hasdai et al. 1998).

Administration of recombinant IGF-I

To determine systemic cardiovascular effects in humans, IGF-I has been injected subcutaneously into healthy men (Donath et al. 1996). Cardiac function and performance were evaluated by echocardiography and an exercise test. IGF-I improved cardiac performance with a significant increase in stroke volume and cardiac output of 14 % and 18 %, respectively. The ejection fraction increased by 9% after IGF-I treatment. Heart rate was not significantly increased at rest or during exercise. Systolic blood pressure was slightly increased by IGF-I, whereas diastolic blood pressure was slightly decreased (Donath et al. 1996). Two years later, the same group published a study with eight patients with chronic cardiac failure; acute administration of IGF-I in patients with chronic heart failure improved cardiac performance by afterload reduction and possible positive ionotropic effect (Donath et al. 1998).

3.5. ATHEROSCLEROSIS AND THE INSULIN-LIKE GROWTH FACTOR AXIS

Although the functions of circulating IGFs have become clearer over the past decade, the actions of locally produced IGFs are still ill-defined. IGF-I, IGF-II, and their binding proteins are secreted by cells of the cardiovascular system. IGFs, IGF-binding proteins and their specific proteases constitute an IGF axis which determines the extent of IGF-dependent cellular effects (Bayes-Genis et al. 2000). Accumulating evidence indicates that IGFs and their regulatory proteins, secreted by the cells of the cardiovascular system, are growth promoters for arterial cells and mediators of cardiovascular disease (Cercek et al. 1991; Ferns et al. 1991; Delafontaine P. 1995; Grant et al. 1996). Atherosclerotic plaque develops over several decades and involves inflammatory cell infiltration, smooth muscle cell proliferation, accumulation of extracellular matrix, fibrous cap formation, and angiogenesis (Ross 1999). Among various growth factors in plaque development, IGFs play a relevant role. The different cell types secrete IGFs, and type I IGF receptors are present on smooth muscle cells (Pfeife et al. 1983), inflammatory cells (Hochberg et al. 1992), and arterial endothelial cells

(Bar et al. 1984) within the atherosclerotic lesion.

Activation of vascular smooth muscle cells (VSMCs)

VSMC dysregulation at atherosclerotic sites is associated with a shift from the contractile to synthetic phenotype and displays many features of growth factor activation (Bayes-Genis et al. 2000). Several studies of both animal (Clemmons 1985; Banskota et al. 1989) and human VSMCs (Grant et al.1994) have shown that IGF-I induces proliferation and migration of these cells .

Studies in ischemic patients reinforce the IGF effects seen *in vitro*. Expression of IGF-I, IGF-I receptor, and IGFBP-1, -2, -3, -4, and -5 has been demonstrated in human coronary atherectomy specimens (Grant et al. 1996). Inhibition of IGF-stimulated coronary smooth muscle cell proliferation by the somatostatin analog octreotide has been substantiated (Grant et al. 1994). Migration of VSMCs from the media is a major pathologic vascular response leading to development and progression of the atherosclerotic lesions. IGFs are potent stimuli for VSMC migration, and the effect appears to be mediated through type I IGF receptor (Bayes-Genis et al. 2000). Interestingly, IGFBP-1 stimulates migration independent of IGF-I via the alpha5beta1integrin (Jones et al. 1993).

Little is known about IGFBPs and proteases in atherosclerosis. IGFBP-2 and IGFBP-4 are the more prevalent binding proteins secreted by VSMCs (Cohick et al. 1993; Gockerman et al. 1995). Recently, a novel IGFBP-4-specific protease was isolated and identified as pregnancy- associated plasma protein-A (Lawrence et al. 1999). By cleaving IGFBP-4 and releasing free IGF-I, PAPP-A appears to modulate growth in local proliferative responses (Conover et al. 1993).

Macrophage activation

Macrophage accumulation is an early event in atherosclerosis. Type I receptors on the macrophage surface allow the IGFs to modulate macrophage concentration at the vessel wall (Hochberg et al. 1992). Human macrophages also synthesize and secrete IGF-I (Nagaoka et al. 1990) and some of the binding proteins (Li et al. 1996). IGF-I has been shown to increase macrophage intake and degradation of low-density lipoprotein (Hochberg et al. 1992).

Angiogenesis

Normal coronary arteries have no vessels within the inner media or intima. Angiogenesis is regulated by many growth factors including fibroblast growth factor, vascular endothelial growth factor, transforming growth factor, and IGF-I (Nicosia et al., 1994; Grant et al., 1993). Endothelial cells possess receptors for IGF-I and secrete IGF-I and IGFBPs (Delafontaine P, 1995). IGF-I has an chemotactic action on vascular endothelial cells (Grant et al., 1987).

Inflammatory angiogenesis occurs in atherogenesis and involves both endothelial cells and macrophages. Animal studies after microembolisation of coronary artery showed alterations in gene expression of IGF and the binding proteins 3, 5, and 6 in macrophages (Kluge et al., 1997).

Restenosis

Coronary restenosis is a major clinical problem after revascularization procedures. Several studies indicate that IGF-I is involved in local cellular events leading to restenosis after

angioplasty (Hansson et al., 1987; Cercek et al., 1991). In the early stages of restenosis , VSMC IGF-I from human restenotic specimens is much higher than in normal coronary VSMCs (Grant et al. 1994). In addition, IGF-I appears to have a growth-promoting effect on VSMCs after balloon injury. VSMC injury has been shown to increase the expression of PAPP-A, an IGFBP-4 protease, which in turn releases free IGF-I and appears to be involved in neointimal hyperplasia (Bayes-Genis et al. 2001).

3.6. CARDIOVASCULAR RISK FACTORS

The concept of risk factors for coronary heart disease (CHD) was established in the Framingham Heart Study report in 1961 (Kannel et al. 1961). Hypercholesterolemia, hypertension, and smoking were identified as major contributors to cardiovascular disease. These findings were confirmed in the Seven Countries Study (Keys et al. 1966). Later the effect of high serum cholesterol in atherosclerosis has been shown to be mainly due to LDL-cholesterol. Research from experimental animals, laboratory investigations, epidemiology, and genetic forms of hypercholesterolemia indicate that elevated LDL cholesterol is a major cause of CHD. The relationship between LDL cholesterol levels and CHD risk is continuous over a broad range of LDL levels, from low to high (NCEP Expert Panel, 2001).

Epidemiological data consistently show a continous, positive, linear relationship of the height of both systolic and diastolic blood pressure with the incidence of coronary heart disease and stroke (Alderman 1993; Hansson et al. 1999).

Cigarette smoking is the third well-established risk factor (Sackett et al. 1968). As nicotine impairs endothelium-dependent vasodilatation in human vessels (Chalon et al. 2000) and cigarette smoke contains a large number of oxidants (Church and Pryor 1984), it has been proposed that the effects of smoking may result from oxidative damage to vascular endothelium (Celermajer et al. 1993). In a cohort study with 7178 elderly persons (65 years of age or older), current smoking increased the risk of total, cardiovascular, and cancer mortality about 2-fold both among men and women (LaCroix 1991).

3.7. INSULIN RESISTANCE AND RISK FACTORS FOR CORONARY HEART DISEASE

As the contribution of classical risk factors to coronary mortality is waning , the importance of obesity, insulin resistence, and NIDDM continues to grow (Braunwald 1997). Insulin resistance can be defined as a decrease in the effect of insulin to stimulate glucose uptake. Postreceptor defects in insulin action lead to compensatory elevation in insulin secretion and and common findings in individuals with insulin resistance are elevated fasting, postprandial and post-glucose insulin levels (Laakso 1996). When the pancreas is no longer able to sustain this compensatory hyperinsulinemia, non-insulin dependent diabetes mellitus with significant hyperglycaemia develops (Reaven et al. 1988).

The hypothesis that elevated insulin levels might contribute to the risk of atherosclerosis was first presented by Nikkilä et al in 1965. In 1979, the first prospective study published on hyperinsulinemia and coronary heart disaese (Pyörälä 1979) initiated a long-lasting debate on the role of hyperinsulinemia in CHD. In 1988, Reaven described hyperinsulinemia and insulin resistance to cluster with several risk factors, such as with glucose intolerance, low HDL cholesterol, high total triglycerides, and hypertension, and suggested that the metabolic and hemodynamic abnormalities associated with insulin resistance play

a major role in the eathiology of CHD. Since then, abundant data has accumulated in support of this hypothesis. To emphasize our inadequate understanding of these relationships, Reaven called the cluster of risk factors syndrome X, which was later replaced by the term metabolic syndrome. The metabolic syndrome is a widespread phenomenon and makes a major contribution to common and serious diseases. The incidence in the middle-aged Finnish population was 17% in men and 8% in women, defined as a clustering of dyslipidemia (hypertriglyceridemia, low HDL-cholesterol or both) and insulin resistance (abnormal glucose tolerance, hyperinsulinemia or both).(Vanhala et al. 1997)

Measurement of insulin sensitivity

Assessing peripheral insulin resistence is difficult. The gold standard for assaying insulin sensitivity has been the euglycemic hyperinsulinemic clamp which involves a constant insulin infusion while maintaining euclycemia by infusing a variable amount of glucose (DeFronzo et al. 1979). This method is technically demanding and limits the number of subjects to be studied. As an alternative methodology, one may use the frequently sampled intravenous glucose tolerance test. Perhaps the simplest measure of insulin sensitivity is the ratio of plasma glucose to insulin. The steady-state basal plasma glucose and insulin concentrations are determined by their interaction in a feedback loop. A computer-based model (homeostasis model assessment HOMA) has been used to predict the homeostatic concentrations that arise from varying degrees of beta-cell deficiency and insulin resistance (Matthews et al., 1985). Hence, a simple but accurate measure of peripheral insulin sensitivity still awaits discovery.

3.8. NEW RISK FACTORS

The past decade has been characterized by growing interest in atherosclerosis as an inflammatory disease (Ross 1999). Several reports have shown a correlation between atherosclerosis and Chlamydia Pneumoniae (Libby et al. 1997), which has been identified in atheromatous lesions of coronary arteries (Jackson et al. 1997). Increased titer of antibodies has been used as a predictor of future adverse events in patients who have had a myocardial infarction (Thom et al. 1991; Gupta 1997). Infection, combined with other factors, may be responsible for the development of atherosclerotic lesions in some patients. In a recent report antimicrobial treatment reduced the risk of cardiovascular events in patients with non-Q-wave infarction or unstable angina (Sinisalo et al. 2002).

Chronic inflammatory responses are often associated with specific types of injurious agents. Inflammation contributes on several levels to the rupture of vulnerable atherosclerotic plaques and to the superficial intimal erosion, both of which may be followed by coronary thrombosis (Libby 2001). Thinning of the fibrous cap is apparently due to the continuing influx and activation of macrophages releasing metalloproteinases and other proteolytic enzymes at these sites. These enzymes cause degradation of the matrix, which may subsequently lead to hemorrhage and thrombus formation (Ross 1999).

A number of inflammatory markers acting independently of myocyte necrosis have been linked to atherosclerosis and acute coronary syndromes. Of these, the most widely studied is C-reactive protein. Elevated levels of C-reactive protein are associated with increased risk of events across the spectrum of coronary syndrome, independent of the presence or absence of myocyte necrosis (Morrow et al. 1998). More recently, C-reactive protein has been implicated as having direct atherothrombotic effects (Pasceri et al. 2000; Zwaka et al. 2001). Interestingly, an association between C-reactive protein and features of the metabolic syndrome has been found (Frohlich et al. 2000; Jonkers et al. 2002) which interrelates

inflammation, lipids and insulin resistance.

In a recent report, Bayes-Genis and colleagues (2001) present histologic evidence that pregnancy-associated protein-A (PAPP-A) is expressed in ruptured but not stable plaques. Circulating PAPP-A levels were significantly elevated in patients with acute coronary syndromes. Elevated PAPP-A appeared to identify patients with unstable angina in the absence of elevated troponin or C-reactive protein levels. It is noteworthy that PAPP-A is a metalloproteinase. The production of metalloproteinases by macrophage foam cells is thought to contribute to the rupture of plaques in patients with acute coronary syndromes (Libby 2001). PAPP-A also regulates IGF-I bioavailability through proteolysis of IGFBP-4. PAPP-A produced by smooth muscle cells has been shown to increase in the coronary after angioplasty suggesting a possible role of PAPP-A in neointimal hyperplasia (Bayes-Genis et al. 2001).

4. AIMS OF THE STUDY

Insulin resistance is an underlying metabolic abnormality involving clustering of cardiovascular risk factors including hypertension, decreased serum HDL, elevated triglyceride concentration, and glucose intolerance. Assessing peripheral insulin resistance is difficult. Insulin resistance is counterbalanced by increased insulin secretion. Previous studies have shown that insulin inhibits IGFBP-1 production in the liver. With this background, the present study was undertaken to evaluate serum IGFBP-1 as a possible indicator of insulin resistance. We also wished to find out whether cardiovascular risk factors or cardiovascular events were associated with serum IGFBP-1.

The specific purposes of our study were:

- To assess the magnitude of the genetic component in the variation of circulating levels of the components of the IGF system (I)

- To study whether serum IGFBP-1 concentration correlates with insulin sensitivity assessed by the insulin-glucose clamp (IV)

- To investigate whether serum IGFBP-1 level correlates with cardiovascular risk factors (II)

- To examine whether serum IGFBP-1 predicts cardiovascular mortality (III)

5. MATERIALS AND METHODS

5.1. SUBJECTS

Table 1. Study subjects

	Subjects		Age (years)
I	79 twin pairs	(32 MZ, 47 DZ)	44-77
II	331 men		70-89
III	622 men		65-84
IV	41 twin pairs	(21 MZ, 20 DZ)	54-72

MZ=monozygotic
DZ=dizygotic

Twins (Studies I and IV)

The Finnish Twin Cohort Study is a program examinig genetic and environmental determinants of chronic disease and risk factors (Kaprio et al. 1978). For the present study, twin pairs resident in Helsinki and the surrounding communities, with no known diabetes, were identified from the Twin Cohort and asked to participate in a study of glucose tolerance. Seventy-nine twin pairs (32 monozygotic, 47 dizygotic) with a mean age of 65 years were included in Study I. A subset of these pairs was further asked to participate in a more detailed study on insulin sensitivity and insulin secretion (Study IV). The final sample size was 41 pairs (21 monozygotic, 20 dizygotic) for the Study IV.

The Seven Countries Study (Studies II and III)

The Seven Countries Study was designed to investigate the incidence of coronary heart disease and associated risk factors in different countries (Keys et al. 1966). The Finnish part of this study included two cohorts selected from geographically defined rural areas: Ilomantsi in eastern Finland (East) and Pöytyä and Mellilä in southwestern Finland (West) (Karvonen et al. 1967). The original cohorts invited to participate in the study in 1959 consisted of all men living in these areas born in 1900-1919 and included 1711 men. Follow-up examinations were performed 5, 10, 15, 25 and 30 years later. Of the original 1959 cohort of 1711 men, 766 were alive in 1984 of which 686 underwent medical examination. Fasting serum samples were available from 622 men, and these formed the study group in 1984. In 1989, 524 men of the original cohort were still alive and were invited to participate in the 30-year examination. 413 men underwent medical examination. A fasting serum sample was available from 331 men and these men formed our study group in 1989. The reasons for nonparticipation included long distance travel, dementia, recent myocardial infarction, severe arthritis, refusal to participate, and inadequate fasting.

5.2. STUDY DESIGNS AND LABORATORY ANALYSES

Study I

Serum samples were obtained from the subjects after overnight (more than 8 h) fasting and stored at -20° C until tested. IGF-I was measured using RIA Kits from INCSTAR Corporation (Stillwater, MN, USA). The assay employs acid treatment and ODS-silica gel extraction to dissociate IGF-I from its binding proteins. This method had a sensitivity of 2.0 nmol/L and the intraassay coefficient of variation (SD/mean) was 10% at the level of 13 nmol/L.

Monoclonal antibody against recombinant hIGF-II (Austral Biologicals, San Ramon, CA) was generated essentially as described earlier for another protein (Koistinen et al. 1994). IGF-II was measured by a competitive immunofluorometric assay using immobilized monoclonal anti-IGF-II antibody and Europium-labeled recombinant human IGF-II. IGF-II was labeled with the Delfia Eu-labeling reagent (Eu-chelate of isothiocyanatobenzyl-diethylene-triaminetetraacetic acid, Wallac, Turku, Finland). For solid-phase coating, rabbit antimouse immunoglobulins (Dakopatts Glostrup, Denmark), 2 μg/200 μL Tris buffer (50 mmol/L Tris-HCl, pH 7.7, containing 9 g/L NaCl and 0.5 g/L NaN_3), were adsorbed onto polystyrene microtiter wells (Eflab, Helsinki, Finland) by an overnight incubation at room temperature. After washing with a washing solution (Tris buffer containing 0.5 g/L Tween 20), the strips were saturated with 1% bovine serum albumin for 2 h. Monoclonal mouse antihuman IGF-II, clone F69-1F6, 40 ng/200 μL assay buffer (Tris buffer containing 5 g/L bovine serum albumin, 0.5 g/L bovine gammaglobulin, 2 g/L diethylenetriaminepentacetic acid and 0.1 g/L Tween 20) were added into the wells and incubated at room temperature. After 1 h the wells were washed twice with washing solution. The standards were recombinant human IGF-II diluted in normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA) and were extracted like the serum samples. The IGFs were separated from the IGFBPs by extraction in formic acid: 50 μL serum and 25 μL 8 mol/L formic acid containing 0.05 % Tween 20 were mixed, and 175 μL ice cold acetone was added, mixed, and centrifuged at 5000 g at 4° C for 15 min. The supernatant (200 μL) was transferred to another tube, evaporated under nitrogen for 0.5 h, then dissolved in 200 μL distilled water and lyophilized. The lyophilized sample was suspended in 120 μL assay buffer. In the assay, 25 μL sample and 150 μL assay buffer were added into the antibody- coated microtiter wells, and, after overnight incubation at 4° C, Europium-labeled IGF-II (2 ng/50 μL) was added to the wells and incubated for another 40 minutes at room temperature. After washing four times, 200 μL enhancement solution (0.1 mol/L acetate phthalate buffer, pH 3.2, containing 0.1 ml/L Triton X-100, 15 μmol/L 2-naphthoyl trifluoroacetone and 50 μmol/L tri-n-octylphosphine oxide; LKB Wallac, Turku, Finland) was added. The wells were shaken gently for 2 min and the fluorescence was measured by the Delfia research fluorometer Model 1234 (LKB Wallac). Sensitivity of the IGF-II assay was 120 μg/L, intraassay coefficients of variation were 7.2% at 806 μg/L and 8.6% at 406 μg/L, and interassay coefficients of variation were 4.9% at 779 μg/L and 13% at 379 μg/L. For analytical recovery, IGF-II was added to serum and four separate extractions were assayed in duplicate. Analytical recovery was 75-125%. Crossreactivities were tested with 1 mg/mL of IGF-I (Austral Biologicals), IGFBP-1 (Koistinen et al., 1990), IGFBP-3 (Celtrix Pharmaceuticals Inc., Santa Clara, Ca, USA) and IGFBP-2,-4,-5,-6 (Austral Biologicals). IGFBPs 1 to 6 and IGF-I did not crossreact in the IGF-II assay when added directly to the immunoassay, nor did they affect the IGF-II levels when added to serum before extraction.The IGF-II standard curve and the dilution curve of extracted serum were parallel. Normal serum levels of IGF-II were 653±126 μg/lL (Mean±SD, range 353-909 μg|L, n=34).

The fasting serum IGFBP-1 concentration was determined by a two-site immunofluorometric assay using two monoclonal antibodies, F34-15C9 and F36-9G3, as described earlier (Koistinen et al. 1996). The intraassay variation was 3-11%, the interassay variation was 4-10%, and the sensitivity of the assay was 0.1 μg/L.

IGFBP-3 was determined from serum samples using monoclonal antibodies (mAb) generated against recombinant IGFBP-3 E. Coli (Celtrix Pharmaceuticals) (Koistinen et al. 1994). The assay used mAb F42-1B6 as the solid-phase antibody and mAb F41-5C11 as the Eu-labeled tracer. The intraassay coefficient of variation was 3.6-6.2% and the interassay coefficient of variation 5.4-11%. The assay had no crossreactions with the other human IGFBPs or the IGFs .

Study IV

After an overnight fast, blood samples were drawn for the analysis of plasma glucose, serum insulin, and insulin-like growth factor binding protein-1 concentrations. Height and weight were recorded and body mass index (BMI) was calculated as weight/height2 (kg/m2). Waist circumference and hip circumferences were measured. As a measure of abdominal obesity, waist-hip ratio (WHR) was calculated. Insulin secretion was measured by the use of an intravenous glucose tolerance test (IVGTT) during which 0.3 g/kg (maximum dose 35g) of 50% glucose was infused intravenously for 2 min and blood samples for the analysis of plasma glucose and serum insulin were drawn at 0, 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 minutes. The first-phase insulin secretion (FPI) was calculated as the incremental area under the insulin curve during the first 10 minutes and the late-phase insulin secretion (LPI) as the incremental area under insulin curve during the last 50 min of the IVGTT. Whole body glucose uptake was quantified with a 160-min euglycemic hyperinsulinemic (45 mU/m2) clamp as previously described (Eriksson et al., 1989). The IVGTT and the clamp were performed on separate days in 14 MZ and 5 DZ twins and on the same day in 7 MZ and 15 DZ pairs. When done on the same day, the clamp was started 30 min after the end of IVGTT. Both members in a twin pair participated in the same clamp protocol. Plasma glucose was measured on duplicates with a glucose oxidase method using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA). Serum insulin concentrations were measured by radioimmunoassay (Pharmacia, Uppsala, Sweden) with an interassay CV of 5%. Serum IGFBP-1 was measured as described previously for Study I.

Studies II and III

The 25-year follow-up study in 1984 and the 30-year follow-up study in 1989 included questionnaires, physical performance measurements, and laboratory investigations. Glucose tolerance was tested using a 75-g oral glucose load. In 1984, the participants were requested to fast for 4 h before arrival. Between 8.00 am and 4.30 pm fasting and 2-h post-load capillary blood glucose levels were measured by a refractometric method (Glucometer, Miles Laboratories, Elkhart, IN, USA). In 1989, the test subjects were asked to fast for at least 12 hours, and the test took place between 8.00 and 12.00 am. Blood glucose was measured from venous plasma using the glucose dehydrogenase method (Glucose Analyzer II, Beckman Instruments, USA). The individuals were hierarchically classified according to the WHO criteria for diabetes mellitus and impaired glucose intolerance (WHO 1985). Insulin analyses were performed using the Pharmacia Diagnostica Phadeseph Insulin RIA kit (Pharmacia, Uppsala, Sweden) at fasting and 2 h after the glucose load.

Total and HDL-cholesterol concentrations were analyzed from fresh sera by an enzymatic method (Monotest, Boehringer Mannheim GmbH, Germany) using an Olli C 3000 photometer (Kone Ltd. Espoo, Finland). HDL was measured after precipitation of VLDL and LDL by the dextran-magnesium-chloride method (Kostner 1976), and serum triglycerides after enzymatic hydrolyzation and determination of the liberated glycerol by colorimetry with commercial agents (GPOPAP method, Boehringer Mannheim GmbH, Germany) using KONE C automatic discrete analyzer.

The serum samples were kept frozen at -20° C until tested, after establishing that repeat freezing, thawing, and storage do not affect the IGFBP-1 level (Rutanen et al. 1984). The IGFBP-1 concentration was determined by a specific immunofluorometric assay using two monoclonal antibodies, F34-15C9 and F36-9G3 (Koistinen et al. 1996). The sensitivity of the assay was 0.1 μg/L, intraassay variation 3-11%, and linear measuring range 0.1-100 mg/L. The IGF-I concentration was determined by ELISA (Diagnostic Systems Laboratories, Webster, TX, USA). The inter- and intraassay coefficients of variation were 4.8-8.8% and 4.5-7.1%, respectively. The IGFBP-3 concentration was determined with immunofluorometric assay (Koistinen et al. 1994). Inter- and intraassay coefficients of variation were 4.9-11% and 3.6-6.2%, respectively, and the detection limit was 0.3 μg/L. Serum insulin, uric acid, triglyceride, IGF-I, and IGFBP-3 concentrations were determined from the samples taken in 1989 only.

Mortality data during follow-up (Study III)

Mortality data were systematically collected during periodic visits to the areas and with the aid of computerized data linkage to the Finnish Death Register. Death certificates and hospital records were collected for all the men who died after the 25-year follow-up examination in 1984 until 1.1.1995. The final cause of death was adjudicated by a single reviewer in order to minimize variability in allocation of the causes of death. In 1995, the vital status of all subjects was ascertained through the Finnish Population Registry. In addition, for the study population, all hospital discharge diagnoses with ICD-8 codes 410-414 and 424-440 were identified from the National Hospital Discharge Register, and hospital records were collected and reviewed. The coder of causes of death was blind to the risk factor status of the subject. Where multiple causes of death were recorded, priority was given to accidents, advanced-stage cancer, CHD, stroke, and other conditions as listed. The basic coding was carried out using the Eighth Revision of the WHO International Classification of Diseases (WHO 1967), but later, internal study codes were amplified for a more compact classification (Keys et al., 1966). As outcome parameters, we used total, cardiovascular, and coronary heart disease mortality.

5.3 STATISTICS

Study I

The results are expressed as mean±SD. For IGFBP-1, logarithmic transformation was carried out before statistical analyses to correct for skewness. Statistical analyses were based on standard methods used for twin data. To estimate genetic and environmental components of variance of the traits, standard univariate twin analyses were carried out (Williams et al., 1992). These included the test of homogeneity of the mean values and variances across the twin type. Maximum likelihood analyses based on sample covariance matrices were used to estimate the components of variance (Neale and Cardon, 1992; Williams et al., 1992).

Study II

The results are expressed as mean±SD. Pearson correlation coefficients between IGFBP-1, IGFBP-3, IGF-I, and a large number of cardiovascular risk factors and parameters of insulin resistance syndrome were calculated. Although the distribution of some variables was slightly skewed to the right, logarithmic transformation did not improve the fit, and therefore, the original measured values were used for the statistical analyses. Significance was assumed when two-sided $p<0.05$. Statistical analyses were performed with the SAS statistical software.

Study III

Serum IGFBP-1 levels in 1984 and 1989 were divided into quartiles. High serum IGFBP-1 (≥75th percentile) was entered as a dummy variable (with the three lowest quartiles serving as the reference group) into forced logistic regression models. Age and other coronary risk factors (weight, BMI, smoking, systolic blood pressure, hypertension, antihypertensive treatment, serum cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, impaired glucose tolerance, diabetes, fasting serum insulin) were also included in the models as covariates. The risk factor-adjusted risk ratios were estimated as the antilogarithm of coefficients from multivariate models. The 95% confidence intervals were estimated based on the assumption of asymptotic normality of the estimates.

Study IV

Data are expressed as mean±SD. Insulin and IGFBP-1 values were log-transformed because of their skewed distribution. Intraclass correlation coefficients (intraclass r) were calculated using residuals of trait values after adjustment by linear regression of age and sex. Differences between zygosity groups were calculated with two-tailed T-test. Pearson´s correlation coefficients were calculated for the phenotypic correlation using a SOLO statistical package (Biomedical Data Processing, Los Angeles, CA, USA). The influence of interdependence of the twin pair members in the estimation of the significance of cross-twin cross-trait-correlation was controlled for by using methodologies for clustered data, included in the Stata7-statistical package (Stata Corporation, TX, USA).

5.4. ETHICAL ASPECTS

All subjects had given their informed consent, and the study designs were approved by the local Ethics Committees Review Boards.

6. RESULTS

6.1. IGF-I, IGF-II, IGFBP-1, AND IGFBP-3 IN TWINS (I)

A total of 32 monozygotic (MZ) and 47 same-sex dizygotic (DZ) twin pairs were included, of which 42 were female and 37 male pairs. Women had higher IGF-II levels than men, 653±175 vs 522±144 μg/L(mean±SD), respectively ($p=0.0001$). The IGFBP-3 levels were also significantly higher in women compared to men (5441±1018 vs 4496±1084 μg/L, $p=0.001$). The differences remained significant after adjustment for age. IGF-II and IGFBP-3 values were adjusted for gender and age prior to the twin analyses. The intrapair correlations for the IGF-I levels were $r=0.41$ ($p=0.009$) for MZ twins and $r=0.12$ ($p=0.22$) for DZ twins of the same sex . The intrapair correlation for the IGF-II concentration was $r=0.66$ ($p=0.0001$) for MZ twins and $r=0.34$ ($p=0.01$) for same-sex DZ twins. The correlations for the IGFBP-3 levels were $r=0.65$ ($p=0.0001$) for MZ pairs and $r=0.23$

(p=0.06) for DZ pairs of the same sex. No significant intrapair correlations were found for the IGFBP-1 concentrations in MZ twins r=0.12 (p=0.2) or DZ twins r=0.18 (p=0.1). The estimated heritability, i.e., the proportion of variance attributable to genetic effects in the best fitting AE-model was 38% for the IGF-I concentration, 66% for the IGF-II concentration, and 60% for the IGFBP-3 concentration. For all three traits, the E model fitted the data poorly (p>0.05), and additional parameters (D or C) did not improve the model fit. No significant heritability was found for IGFBP-1 concentrations.

6.2. IGFBP-1, INSULIN SENSITIVITY AND INSULIN SECRETION IN TWINS (IV)

No correlation was present between IGFBP-1 and first-phase insulin secretion, whereas an inverse correlation between IGFBP-1 and late-phase insulin secretion was found (r=-0.39, p=0.001). The correlation coefficient between IGFBP-1 and LPI was -0.380 for MZ twins and -0.359 for DZ twins. Serum IGFBP-1 correlated positively with insulin-stimulated glucose uptake (r=0.34, p=0.002). The correlation coefficient was 0.407 for MZ and 0.178 for DZ twins. The cross-twin cross-trait correlations between IGFBP-1 and insulin-stimulated glucose uptake were similar for MZ (r=0.10; p=0.54) and DZ (r=-0.08; p=0.55) twins. The cross-twin cross-trait correlation between IGFBP-1 and LPI was higher in MZ (r=-0.27; p=0.06) than DZ (r=-0.06; p=0.68) twins.

6.3. IGFBP-1 AND CARDIOVASCULAR RISK FACTORS (II)

Fasting serum IGFBP-1 concentration correlated positively with age and HDL-cholesterol. Negative correlations were found between IGFBP-1 and insulin, BMI, and serum triglycerides. For comparison, the correlation coefficients for insulin and cardiovascular risk factors were calculated. Glucose tolerance was tested in 300 subjects, of whom 48.3% had normal glucose tolerance, 19.0% had impaired glucose tolerance, and 32.7% had diabetes. In diabetic subjects, a significant correlation between IGFBP-1 and systolic blood pressure was found (r=-0.23; p=0.02). No significant correlation was present between IGF-I and any of the cardiovascular risk factors. Serum IGFBP-3 levels correlated positively with serum cholesterol and negatively with age, serum insulin, and blood glucose.

The homeostasis model assessment was applied in an attempt to evaluate whether insulin sensitivity and/or insulin secretion (beta-cell function) were determinants of the serum IGFBP-1 concentrations. Correlations between serum IGFBP-1 and insulin sensitivity (r= 0.25) and beta-cell function (r=-0.27) were highly significant (p=0.0001 for both). Consequently, 43 % of the subjects belonging to the lowest quartile of serum IGFBP-1 concentrations could also be assigned to the most insulin-resistant quartile.

6.4. IGFBP-1 AND CARDIOVASCULAR MORTALITY (III)

Of the 622 study participants, 358 died between 1 January , 1984 and 1 January, 1995. A total of 160 subjects died from cardiovascular disease, with 113 of them being coronary deaths. A nonlinear association was present between serum IGFBP-1 and total and cardiovascular mortality, with the highest risk in the highest quartile. Variables associated with IGFBP-1 and other putative risk factors were entered in multiple regression analysis. The risk of total and CVD mortality increased with increasing age and smoking. Fasting serum insulin was not a significant predictor for any of the outcome parameters. Furthermore, the effect of high IGFBP-1 was similar in different categories of glucose tolerance

irrespective of whether the WHO criteria or the American Diabetes Association criteria were used. No association was found between IGF-I or IGFBP-3 and any category of mortality. High serum IGFBP-1 (≥75 percentile) in 1984 was associated with increased total mortality (p=0.0002), cardiovascular mortality (p=0.0009), and coronary heart disease mortality (p=0.002) during the 5-year follow-up. After age adjustment, high serum IGFBP-1 remained a significant predictor of death from all causes (p=0.007), from cardiovascular disease (p<0.008), and from coronary heart disease (p=0.01) during the 5-year follow up. In the 10-year follow-up, high serum IGFBP-1 was predictive of death from all causes (p=0.008), from cardiovascular disease (p=0.04), and from coronary heart disease (p=0.01). In the 10 year follow up the trend diminished after adjustment for age but remained significant for death from coronary heart disease (p=0.04).

7. DISCUSSION

7.1. THE GENETIC AND ENVIRONMENTAL FACTORS IN THE VARIATION OF IGF-I, IGF-II, IGFBP-1, and IGFBP-3

In the twin population, a substantial genetic contribution responsible for the variation of circulating levels of IGF-I, IGF-II, and IGFBP-3 was found. The IGF-I and IGFBP-3 levels are age-dependent. Serum IGF-I and IGFBP-3 concentrations normally increase until puberty and decrease thereafter (Hall et al. 1984). We found the intrapair correlation for the IGF-I levels to be much weaker than previously reported in children (Kao et al. 1994). This suggests that age-related factors may affect the expression of the gene. Growth hormone regulates the IGF-I and IGFBP-3 concentrations in serum. In children, relative body height is correlated with serum IGFBP-3 levels (Vihervuori et al. 1996). In our study with adult subjects, no significant correlation existed between IGFBP-3 and height.

As opposed to IGF-I, IGF-II and IGFBP-3, no significant heritability for IGFBP-1 concentrations was found. This is consistent with IGFBP-1 being involved in rapid metabolic adaptation to nutritional stimuli. Thus serum IGFBP-1 appears to be regulated mainly by environmental factors. In a recently published twin study, by contrast, genetic variance accounted for more than 60% of the glucose-stimulated insulin secretion and almost 40% of insulin-stimulated glucose uptake (Lehtovirta et al. 2000).

7.2. IGFBP-1, INSULIN SECRETION, AND INSULIN SENSITIVITY

Cross-twin cross-trait correlation coefficients between IGFBP-1 and insulin secretion were higher in MZ than DZ twins. No difference was seen between MZ and DZ twins in the correlation between IGFBP-1 and insulin-stimulated glucose uptake. This suggests the precsence of a latent, possibly genetic, influencing factor shared by IGFBP-1 and insulin secretion, but not by IGFBP-1 and insulin sensitivity.

Serum IGFBP-1 has been suggested as a useful marker for insulin resistance syndrome . Our experimental protocol combining an IVGTT with a euglycaemic clamp showed significant correlations with both insulin sensitivity and late-phase insulin secretion. However, insulin secretion is dependent upon the degree of insulin sensitivity, and this study cannot distinguish between the effects of insulin sensitivity and secretion on IGFBP-1. Yki-Järvinen and co-workers (1995) studied healthy volunteers and IDDM patients and found that portal insulin rather than insulin sensitivity regulates serum IGFBP-1. They suggested that IGFBP-1 reflects hepatic insulinisation and may be used as a marker for insulin sensitivity only in individuals with intact insulin secretion.(Yki-Järvinen et al.

1995). Using sophisticated methods to measure insulin secretion, impaired insulin secretion has not been detected until the patients have impaired glucose tolerance (Eriksson et al. 1989). Insulin resistance is present in the majority of patients with various states of glucose intolerance, but actual decompensation of glucose homeostasis does not occur if individuals can maintain a state of compensatory hyperinsulinemia (Weyer et al. 1999). Consequently, serum IGFBP-1 may be used as a marker of insulin sensitivity in subjects with intact insulin secretion, and might help in early detection of individuals with insulin resistance.

7.3. IGFBP-1 AND CARDIOVASCULAR RISK FACTORS

The present study on cardiovascular risk factors in elderly men confirmed that the low fasting IGFBP-1 level is associated with a number of cardiovascular risk factors such as decreased serum HDL cholesterol, elevated triglyceride concentration, hyperinsulinemia, and decreased insulin sensitivity. This supports previous findings by Gibson and co-workers (1996), who showed that reduced IGFBP-1 levels correlate with low HDL cholesterol, high insulin, and elevated blood pressure in NIDDM patients. Also in a non-diabetic population, a positive correlation has been found between IGFBP-1 and HDL cholesterol, and a negative correlation between IGFBP-1 and insulin (Janssen et al. 1998).

These findings may be a reflection of insulin resistance and hyperinsulinemia. In the present work, the correlations between IGFBP-1 and HDL-cholesterol and triglycerides are less marked than those between insulin and blood lipids. Interestingly, after adjustment for insulin, the associations between IGFBP-1, HDL and triglycerides failed to retain statistical significance. As mentioned earlier, the production of IGFBP-1 is suppressed by insulin, and low serum IGFBP-1 values reflect high insulin values in the circulation and/or the portal vein.

The observed phenomenon concerning the HOMA estimates can also be explained by insulin levels, although the HOMA model is dependent on accurate insulin measurements and cross-reactivity with proinsulin precursors may result in some inaccuracy in HOMA estimates. One may assume, however, that subjects with low serum IGFBP-1, hyperinsulinemia, hypertriglyceridemia and low HDL cholesterol may be insulin-resistant. Therefore, it is plausible that a reduced circulating level of IGFBP-1 is associated with a group of factors that predispose to cardiovascular morbidity (Gibson et al. 1996; Heald et al. 2001). In conclusion, low serum IGFBP-1 is found to be associated with an unfavourable cardiovascular risk profile and is another sign associated with the insulin resistance syndrome.

7.3 IGFBP-1 AND CARDIOVASCULAR DEATH

Our studies and other investigations show that low serum IGFBP-1 is associated with an unfavourable cardiovascular risk profile. Accumulating evidence from the literature indicates that IGFs and their binding proteins are growth promoters for the arterial cells and might be mediators of cardiovascular diseases (Bayes-Genis 2000). However, no reports are available on the association between IGFBP-1 and cardiovascular events. In our 5-year and 10-year follow-ups we found that high serum IGFBP-1 level (highest quartile) was associated with increased total, cardiovascular, and coronary heart disease mortality in elderly men. The finding was quite unexpected and not in line with our previous findings on the low IGFBP-1 as a sign of unfavourable cardiovascular risk. However, earlier follow-up studies have demonstrated that hyperinsulinism may not be associated with the impaired survival in the aged (Lindberg 1997). After further analyses and discussions, we concluded that the association between IGFBP-1 and cardiovascular death may be a J-shaped curve.

Those subjects with low IGFBP-1 die because they have insulin resistance and the associated traditional cardiovascular risk factors. Subjects with high IGFBP-1 have an even higher risk, but the mechanism can only be speculated upon.

Is it through inhibition of IGF-mediated metabolic or mitogenic actions? In our investigation, no association was present between cardiovascular mortality and circulating IGF-I level. However, we must bear in mind that total IGF-I measured in our study provides only a crude estimate of the biologically active IGF-I. Administration of human recombinant IGF-I to patients has been demonstrated to decrease fasting glucose and triglyceride concentrations and increase insulin sensitivity (Moses et al. 1996). Therefore, one possible explanation is that high serum IGFBP-1 decreases the amount of unbound IGF-I and attenuates the known beneficial metabolic effects of IGF-I. This would be in line with the work of Janssen and co-workers (1998), who reported high free serum IGF-I levels in subjects with decreased presence of arterosclerotic plaques and signs of coronary heart disease. However, going back to own results we failed to detect high triglyceride or glucose levels in the group with high IGFBP-1. Unfortunately, as in many clinical studies on IGFBP-1, our study focused on circulating levels, and therefore cannot distinguish between the effects of IGF-I/IGFBP-1 complexes in serum and interstitial fluid and the effects exerted directly by IGFBP-1.

The IGF axis is complex and many possible interactions account for the functional diversity the extent of which is only beginning to be understood. Alterations in the balance of components of the IGF axis in the vessel wall influence the cell growth, survival, migration, and extracellular matrix synthesis which modulate the atherosclerotic plaque progression and neointimal formation of restenosis. Improved insight into the IGF axis dynamics could identify new targets to limit or prevent vascular pathologies. (Bayes-Genis A et al., 2000)

A limitation of our study is that our cohort comprised men aged 65-84 years at baseline. Although those who participated are considered representative of their age group, with a high participation rate and complete ascertainment of deaths , by definition, they represent only those men who have survived to old age. Because many cardiovascular events occur at a younger age, these findings must be confirmed in middle-aged men and women.

Our results indicate that high serum IGFBP-1 is associated with an increased risk of coronary heart disease, cardiovascular disease, and total mortality in elderly men. As the reasons for this observation are not obvious further studies are needed to find determine the mechanism. Another interesting approach would be to study the effect of cardiovascular medication, such as statin treatment, on the components of the IGF axis.

8. CONCLUSIONS

- Serum IGFBP-1 is regulated mainly by environmental factors.

-Serum IGFBP-1 may be used as a marker of insulin sensitivity in subjects with intact insulin secretion, and might help in the early detection of insulin-resistant individuals.

-Low serum IGFBP-1 is associated with an unfavourable cardiovascular risk profile and is another sign associated with the insulin resistance syndrome.

-High serum IGFBP-1 is associated with an increased risk of coronary heart disease, cardiovascular and total mortality in elderly men.

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